FORMPTO-1390(Modified) (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR
3027.00020	10/009891

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/CA00/00540	May 10, 2000	May 10, 1999

TITLE OF INVENTION
METHOD FOR MEASURING BIOMOLECULES

APPLICANT(S) FOR DO/EO/US
Ronald Marquardt et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☒ Certificate of Mailing by Express Mail
23. ☐ Other items or information:

CERTIFICATE OF MAILING BY "EXPRESS MAIL"

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Date of Deposit 10/26/01

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office To Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Angel Webb

(Signature of person mailing paper or fee)

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
10/009891	PCT/CA00/00540	3027.00020

24. The following fees are submitted:.

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :		CALCULATIONS PTO USE ONLY
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO	$1040.00	
☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO	$890.00	
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO	$740.00	
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4)	$710.00	
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4)	$100.00	
ENTER APPROPRIATE BASIC FEE AMOUNT =		**$890.00**
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).		**$0.00**

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	17 - 20 =	0	x $18.00	**$0.00**
Independent claims	2 - 3 =	0	x $84.00	**$0.00**
Multiple Dependent Claims (**check if applicable**).			☐	**$0.00**
TOTAL OF ABOVE CALCULATIONS =				**$890.00**
☒ Applicant claims small entity status. See 37 CFR 1.27). The fees indicated above are reduced by 1/2.				**$445.00**
SUBTOTAL =				**$445.00**
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +				**$0.00**
TOTAL NATIONAL FEE =				**$445.00**
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (**check if applicable**). ☐				**$0.00**
TOTAL FEES ENCLOSED =				**$445.00**
			Amount to be: refunded	$
			charged	$

a. ☒ A check in the amount of **$445.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **11-1449** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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Kenneth I. Kohn
NAME

30,955
REGISTRATION NUMBER

October 26, 2001
DATE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application:

RONALD MARQUARDT et al.

National Phase of: PCT/CA00/00540

International Filing Date: May 10, 2000

Attorney Docket Number:3027.00020

Group Art Unit: Examiner:

For: METHOD FOR MEASURING BIOMOLECULES

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

Please preliminarily amend the above-captioned patent application prior to examination on the merits as follows:

AMENDED VERSION

IN THE SPECIFICATION:

Page 1, after the Title, please insert the following section:

CROSS REFERENCE TO RELATED APPLICATIONS

This patent application is a National Phase Concerning a Filing Under 35 U.S.C 371, claiming the benefit of priority of PCT/CA00/00540, filed May 10, 2000, which claims the benefit of priority of Canadian Serial Number 2,270,639, filed May 10, 1999, all of which are incorporated herein by reference.

REMARKS

The above amendment added no new matter and is merely made to more accurately describe and claim the invention, and to claim benefit of priority.

It is respectfully submitted that the application is now in condition for allowance, which allowance is respectfully requested.

Respectfully submitted,
KOHN & ASSOCIATES

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Angel Webb

VERSION SHOWING CHANGES

IN THE SPECIFICATION:

Page 1, after the Title, please insert the following section:

--CROSS REFERENCE TO RELATED APPLICATIONS

This patent application is a National Phase Concerning a Filing Under 35 U.S.C 371, claiming the benefit of priority of PCT/CA00/00540, filed May 10, 2000, which claims the benefit of priority of Canadian Serial Number 2,270,639, filed May 10, 1999, all of which are incorporated herein by reference.--

A METHOD FOR MEASURING BIOMOLECULES

FIELD OF THE INVENTION

The present invention relates generally to the field of enzyme assays.

BACKGROUND OF THE INVENTION

It is of great importance in all field and disciplines of the life sciences to utilize the appropriate qualitative and quantitative analytical techniques for the detection, identification, and measurement of the concentration of a wide variety of biologically important molecules. These analytical techniques can be utilized in many different types of assays including those for enzymes, receptors, lectins and inhibitors, etc.

All chemical reactions in living systems are virtually catalyzed by enzymes, and the assay of enzyme activity is probably one of the most frequently encountered procedures in biochemistry. Most enzyme assays are carried out for the purpose of estimating the amount or activity of an enzyme present in a cell, tissue, other preparation, or as an essential part of an investigation involving the purification of an enzyme. The current assay methods have been developed based on the physical, chemical and immunological properties where they can be detected using photometric, radiometric, high performance liquid chromatographic, electrochemical assays, etc. (Eisenthal, R. and Danson, M. J., 1993). Although the methods basically fulfill the many essential requirements for routine analysis, there are, among those, the varying disadvantages of low sensitivity (Brenda Oppert et al, 1997), multiple steps (Twining, S. S., 1994; Pazhanisamy, S. et al, 1995) and

steps that are tedious and time-consuming (Fields, R., 1976). Immunoassays have been widely using in human clinical tests and therapeutics, agriculture, food, veterinary and environmental diagnostics (Deshpandes, S. 1996). In the most cases, immunoassays are effective and valid (Cleaveland, J. S. et al 1990), but in some cases they are not suitable, for example, in the determination of enzyme activity. This occurs because the binding assays for antibody and antigen (enzyme) can only be used to measure the concentration of an antigen (enzyme) but not its activity. It is of important to know the catalytic activity of an enzyme and not just the amount of the enzyme as a given amount of the enzyme may have a widely varying activity depending on reaction conditions. Also antibodies tend to react only with structurally similar antigens such as a specific enzyme. Therefore, it is often not possible to quantitate the amount of an enzyme from a related species using immunoassays.

Pharmaceutical industries usually utilize conventional methods mentioned above to screen compounds for discovering drugs. This process is slow due to the several steps required and the large amount of compounds needed to be tested; on a good day, a lab might test 100 to 1,000 compounds. In the race to commercialization, pharmaceutical manufacturers are facing great pressure to reduce the time to discover new clinical drugs, cut assay costs, and screen more compounds and against more targets. Therefore, there is a very high demand to develop new methods to meet the requirements of a high throughput screening (HTS). Jones et al (1997) described a method using quenched BODIPY dye-labeled casein as a substrate for determining the activities of protease, which is sensitive and amenable to automation. The degree of quenching of the fluorescent

tag is crucial in this method. If there is not enough quenching due to poor conjugate or degradation of the fluorescence-labeled substrate under storage, etc. the assay will not be very useful. Also this procedure has relatively high background values which reduce its sensitivity. Another example of a potentially useful high throughput assay was made by Marquardt, et al (PCT/US97/07983). The method involves many steps of coating wells of a microplate, washing the wells, adding biologically active substance to wells, washing the wells once more, adding the indicator enzyme to wells, washing the wells again and adding a color development reagent. The assay cannot be readily used in assays requiring rapid analysis.

A new assay method not only having potentially excellent sensitivity but being suitable for high throughput assays is preferable. This invention outlines a procedure that can achieve these goals.

SUMMARY OF THE INVENTION

According to a first aspect of the invention, there is provided a method for measuring the activity or concentration of a bioactive molecule comprising: coating a reaction vessel with a reactant, said reactant being capable of interacting with a bioactive molecule having a biological activity; adding a sample to the reaction vessel, said sample comprising a known quantity of a detectable label and the bioactive molecule having the biological activity; incubating the reaction vessel under conditions wherein the reactant and the sample interact; removing a quantity of detectable label from the sample by binding detectable label to the reactant coated on the reaction vessel; transferring a soluble portion of the sample from the reaction

vessel to a counting vessel ; and measuring the quantity of detectable label in the counting vessel.

The detectable label may be selected from the group consisting of: enzyme label; colorimetric label; radioactive label; luminescent label and fluorescent label.

The sample may be a biological sample.

The biological activity may be an enzymatic activity or a binding affinity.

The sample may include an inhibitor of the biological activity of the bioactive molecule or a competitor of the biological activity of the bioactive molecule.

The bioactive molecule may be selected from the group consisting of: an enzymatic product; an enzyme; a substrate; a lectin; a lectin-binding ligand; a receptor; an inhibitor; a receptor binding ligand; an antigen; and an antibody.

The compound may be selected from the group consisting of: an enzymatic product; an enzyme; a lectin; a lectin-binding ligand; a substrate; a receptor; an inhibitor; a receptor binding ligand; an antigen; and an antibody.

According to a second aspect of the invention, there is provided a method for measuring the activity or concentration of a bioactive molecule comprising: coating a reaction vessel with a reactant, said reactant being capable of interacting with a bioactive molecule, said reactant including a detectable label; adding to the reaction vessel a sample, said sample including the bioactive molecule having a biological activity; releasing a quantity of detectable label from the reactant by incubating the reaction vessel under conditions such that the reactant and the detectable label contact the bioactive molecule and interact with the bioactive molecule; transferring a soluble portion of the sample containing released label from

the reaction vessel to a counting vessel; and measuring the quantity of detectable label in the counting vessel.

The detectable label may be selected from the group consisting of: enzyme label; colorimetric label; radioactive label; luminescent label; and fluorescent label.

The sample may be a biological sample.

The biological activity may be an enzymatic activity or a binding affinity.

The sample may include an inhibitor of the biological activity of the bioactive molecule or a competitor of the biological activity of the bioactive molecule.

The bioactive molecule may be selected from the group consisting of: an enzymatic product; an enzyme; a substrate; a receptor; a receptor ligand; an antigen; a lectin; a lectin-binding ligand; a ligand; and an antibody.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned hereunder are incorporated herein by reference.

DEFINITIONS

"Ligand" as used herein refers to a bioactive molecule having specific binding affinity for another bioactive molecule.

"Receptor" as used herein refers to a bioactive molecule that has a

specific binding affinity for another bioactive molecule, for example, a ligand.

"Bioactive substance", "bioactive molecule" or "biologically active substance" as used herein except where otherwise stated refers to a molecule or complex having a biological activity, for example, an enzymatic activity or binding affinity for another biomolecule.

Described herein is a new method for the qualitative and quantitative analysis of bioactive substances. The assay method is based on the principle of separating the reactants from the products after the completion of the reaction, followed by measurement of the amount of label transferred, as described below. It is important to note however that the method does not involve time-consuming separation steps, such as filtration or centrifugation, nor is it necessary to add reaction-stopping chemicals or agents, meaning that the method is ideally suited for high through-put assays.

The method involves coating a reaction vessel with a reactant, identified hereafter as the coated reactant. Additional reactants are then added to the coated reaction vessel, forming a reaction mix. It is of note that as a result of this arrangement, the reaction mix has a bound portion (the coated reactant) and a soluble portion (the other reactants). Generally, the method is arranged such that either the coated reactant includes a label that will be released by the activity of the bioactive molecule or the soluble portion of the reaction mix includes a label which is bound to the coated reactant as a result of the activity of the bioactive molecule. Thus, label is either transferred from the bound portion of the reaction mix to the soluble portion of the reaction mix or from the soluble portion to the bound portion as a result of the activity of the bioactive molecule. Once the

reaction has been allowed to proceed for a set period of time, the reaction is stopped by removing the soluble portion of the reaction mix from the coated reaction vessel and transferring the soluble reaction mix to a counting vessel, that is, an unused, untreated vessel, wherein the amount of label in the reaction mix is counted. Thus, the amount of the label in the counting vessel is either directly proportional to or reciprocally proportional to the activity or amount of the bioactive substrate, depending on the experimental design, as described below.

Thus, the above-described method can be used to measure the activity of a variety of enzymes as well as the concentration of ligands within a sample, as described below. The method can also be used to measure the effect of various inhibitors on the activity of the enzymes and/or ligands. Furthermore, any suitable detectable label known in the art may be used, as described below. These include, but are by no means limited to, for example, colorimetric labels, radioactive labels, luminescent labels and fluorescent labels.

The invention will now be described by way of examples. However, the invention is not limited to the examples.

EXAMPLE I – TRANSFER OF LABEL FROM SOLUBLE PORTION TO COATED REACTANT

In this example, the surface of the reaction vessel is coated with the coated reactant and the soluble portion of the reaction mix includes at least a bioactive molecule having a biological activity and a labeled substrate for the bioactive molecule. Specifically, the substrate is labeled such that the activity of the bioactive molecule transfers the label from the substrate to the coated reactant,

thereby producing labeled coated reactant. That is, the label is transferred from the soluble portion of the reaction mix to the bound portion of the reaction mix. After a set period of time, the soluble portion of the reaction mix is removed from the coated reaction vessel, transferred to the counting vessel and counted. It is of note that the amount of label remaining in the soluble portion of the reaction mix is reciprocally proportional to the activity of the bioactive molecule.

In an illustrative example, the activity of protein kinase A is assayed. It is of note that in this example, the reaction vessel and the counting vessel are 96-well microplates are from VWR Canlab. As will be appreciated, other suitable vessels may also be used. Specifically, hydrolyzed and partially dephosphorylated casein is dissolved in PBS to the concentration 5 ug/ml, and 100 ul/well is added to each well of the reaction vessel. The reaction vessel is then incubated at 37^0 C for 3 hr, and is then rinsed with PBST, producing a coated reaction vessel. Thus, in this example, the coated reaction vessel is coated with casein (the coated reactant). A series of concentrations of protein kinase A in phosphate buffer (PB pH 7.2, cAMP, ^{32}P-ATP) are then added to the wells of the coated reaction vessel (100 ul/well). Thus, prior to incubation, the coated casien comprises the bound portion of the reaction mix, the labeled ATP, protein kinase A and buffer comprise the soluble portion of the reaction mix and the bioactive molecule is protein kinase A. The reaction mixture is then incubation at 37^0 C for 30 min, during which time a quantity of the label, ^{32}P, is transferred from ^{32}P-ATP to casein by protein kinase A. After a predetermined period of time, the soluble portion of the reaction mix is transferred from each well of the coated reaction vessel to a corresponding well in the counting vessel, thereby stopping the reaction. The radioactivity of ^{32}P-ATP in

the wells of the counting vessel are counted in a scintillation counter. As discussed above, the amount of label remaining in the soluble portion and transferred to the counting vessel is reciprocally proportional to the activity of the protein kinase A.

EXAMPLE II – TRANSFER OF LABEL FROM SOLUBLE PORTION TO COATED REACTANT IN PRESENCE OF INHIBITOR

In other examples, the reaction may be carried out generally as described in Example I with the exception that the soluble portion of the reaction mix also includes an inhibitor of the activity of the bioactive molecule. Thus, in these examples, the amount of label transferred to the counting vessel is directly proportional to the amount of inhibitor present in the reaction mix.

In an illustrative example, the coated reaction vessel is prepared as described in Example I. Varying concentrations of a protein kinase A inhibitor, cAMP, in the phosphate buffer (PB pH 7.2, cAMP, ^{32}P-ATP) are added to the wells (50 ul/well) of the coated reaction vessel. Negative and positive control are also included. A pre-determined amount of protein kinase A (50 ul/well) is added to each well and the coated reaction vessel is incubated at 37^0 C for 30 min. The reaction is halted by removing the soluble portion of the reaction mix and transferring same to wells of the counting vessel. As discussed above, protein kinase A catalyzes the transfer of ^{32}P from ^{32}P-ATP to casein; however, in this example, cAMP inhibits the enzymatic activity of protein kinase A, meaning that the amount of label remaining in the soluble portion and transferred to the counting vessel is directly related to the concentration of the inhibitor.

EXAMPLE III – TRANSFER OF LABEL FROM COATED REACTANT TO

SOLUBLE PORTION

In other examples, the reaction vessel is coated with a labeled coated reactant and the reaction mix includes at least a bioactive molecule having a biological activity. Specifically, the coated reactant is labeled such that the activity of the bioactive molecule causes the label to be released from the coated reactant. That is, label is transferred from the bound portion of the reaction mix to the soluble portion of the reaction mix. After a set period of time, the soluble portion of the reaction mix is removed from the coated reaction vessel and transferred to the counting vessel. Thus, in this example, the amount of label present in the soluble portion of the reaction mix that is transferred to the counting vessel is directly proportional to the activity of the bioactive molecule.

In an illustrative example, the activity of a variety of proteases is measured using a fluorescent label. In this example, the coated reaction vessel and the counting vessel are 96-well microplates are from VWR Canlab, although other suitable vessels may also be used. Specifically, fluo-casein is prepared by mixing 5 mg NHS-coumarin in 100 ul DMSO with 10 mg casein in PBS (pH 7.2) in a micro-centrifuge tube. The tube is then incubated at room temperature for 3 hr. The fluo-casein (coated reactant) is then dissolved in PBS to the concentration 5 ug/ml and 100 ul/well is added to each well of the reaction vessel. The reaction vessel is incubated at 37^0 C for 3 hr, then rinsed with PBST, thereby producing a coated reaction vessel. A series of concentrations of a protease in a buffer (100 ul/well) is then added to the wells of the coated reaction vessel and the coated reaction vessel is incubated at 37^0 C or room temperature for 30 min. During this time, the fluo-casein is hydrolyzed by the protease, releasing the fluorescent label

into the soluble portion of the reaction mix. The soluble portion of the reaction mix is then transferred from individual wells of the coated reaction vessel to corresponding wells of the counting vessel, thereby stopping the reaction. The fluorescent intensity of the label in each of the wells of the counting vessel is measured with a fluorometer. Thus, the amount of label in each well of the counting vessel is directly proportional to the activity of the protease.

Examples of suitable proteases include, but is by no means limited to, for example, proteinase K, elastase, protease XIII, papain, trypsin, pepsin and casein.

EXAMPLE IV - TRANSFER OF LABEL FROM COATED REACTANT TO SOLUBLE PORTION IN PRESENCE OF INHIBITOR

In other examples, the reaction may be carried out as described in Example III except that the soluble portion of the reaction mix also includes an inhibitor of the bioactive molecule. In this example, the amount of label transferred to the soluble portion and subsequently to the counting vessel is reciprocally proportional to the amount of inhibitor present in the reaction mix.

In this example, the coated reaction vessel is prepared as described in Example III. Varying amounts of a protease inhibitor in the buffer (50 ul/well) are added to the wells of the coated reaction vessel. Negative and positive controls are included. A fixed concentration of the protease in buffer (50 ul/well) is added to the wells of the coated reaction vessel. The coated reaction vessel is incubated at 37^0 C or room temperature for 1 hr. The fluo-casein is cleaved by the protease, thereby releasing the fluorescent label into the soluble portion of the reaction mix;

however, this cleavage is impeded by the inhibitor. Thus, the amount of label released into the soluble portion is reciprocally proportional to the degree of inhibition. Following incubation, the soluble portion of the reaction mix is transferred from each of the wells of the coated reaction vessel into corresponding wells of the counting vessel, thereby stopping the reaction. The fluorescent intensity of the label in the wells of the counting vessel is measured with a fluorometer and, as discussed above, is reciprocally proportional to the amount of inhibition of the protease activity.

EXAMPLE V – ENZYMATIC PRODUCTION OF LABELED LIGAND

In other embodiments, the reaction vessel is coated with a coated reactant which is a binding ligand for an enzymatic product, wherein the enzymatic product is formed by a bioactive molecule acting on at least a first substrate and a label. Thus, the activity of the bioactive molecule causes free label to be combined with the first substrate to form an enzymatic product. The enzymatic product then binds to the coated reactant and is removed from the soluble portion of the reaction mix. After a set period of time, the soluble portion of the reaction mix is removed from the coated reaction vessel and transferred to the counting vessel. Thus, in these examples, the amount of label remaining in the soluble portion of the reaction mix is reciprocally proportional to the activity of the bioactive molecule.

In an illustrative example, telomerase activity is measured. Specifically, Streptavidin or avidin (coated reactant) is dissolved in a buffer to a concentration of 5 ug/ml and 100 ul/well is added to each well of the reaction vessel. The reaction vessel is incubated at 37^0 C for 3 hr, and is then rinsed with

PBST, thereby producing a coated reaction vessel. A series of concentrations of telomerase in a reaction mixture containing 50 mM Tris-acetate pH 8.5, 50 mM potassium acetate (KAc), 5 mM β-mercaptoethanol, 1mM spermidine, 1mM $MgCl_2$, 0.5-2 mM dATP, 0.5-2 mM dTTP, 1.5 uM fluo-dGTP, 1 uM biotin-oligonucleotide primer $(TTAGG)_3$, are added to the wells of a the coated reaction vessel and the mixture is incubated at 30^0 C for 1 hr. The DNA synthesis reaction is stopped by adding a stop solution (10 mM Tris-HCl, pH7.5, 230 mM EDTA and 100 ug/ml RNase) and incubating further at 37^0 C for 15 min. In this arrangement, the biotinylated primer binds to the coated reactant. Telomerase then elongates the primer, incorporating fluo-dGTP into the growing oligonucleotide chain. Unincorporated fluo-dGTP remains in the soluble portion of the reaction mixture and is transferred to the counting vessel. The fluorescent intensity of fluo-dGTP transferred into the counting vessel is measured with a fluorometer and is reciprocally proportional to the activity of telomerase. In other embodiments of this example, the telomerase reactions may be carried out in a separate vessel and then transferred to the coated reaction vessel. It is of note that in these embodiments, the telomerase reaction mix transferred to the coated reaction vessel will include both incorporated and unincorporated fluo-dGTP; however, the biotinylated primer will bind to the avidin or streptavidin, thereby removing the incorporated fluo-dGTP from the soluble portion of the reaction mix.

EXAMPLE VI – ENZYMATIC PRODUCTION OF LABELED LIGAND IN PRESENCE OF INHIBITOR

In other examples, the reaction may be carried out as described in

Example V wherein the soluble portion of the reaction mix also includes an inhibitor of the bioactive molecule. As a result of this arrangement, the amount of label transferred to the counting vessel is directly proportional to the amount of inhibitor present in the reaction mix.

In an illustrative example, the coated reaction vessel is prepared as described in Example V. Varying amounts of 7-deaza-dATP in reaction buffer (50 ul/well) are added to the wells of the coated reaction vessel. A fixed activity of telomerase in reaction buffer (50 ul/well) is added to the wells of the coated reaction vessel containing the inhibitor and the controls. The reaction vessel is then incubated at 30^0 C for 1-2 hr prior to stopping the DNA synthesis with the stop solution. As discussed above, the biotin primer binds to the streptavidin (avidin)-coated coated reaction vessel, meaning that incorporated label is removed from the soluble portion of the reaction mix, as discussed above. The soluble portion is then transferred to a counting vessel and the fluorescent intensity of fluo-dGTP is measured with a fluorometer and is directly proportional to the amount of the inhibitor. It is of note that, as discussed above, the telomerase reactions may be carried out in a separate reaction vessel first, as described above.

EXAMPLE VII – BINDING COMPETITION ASSAY

In other examples, the reaction vessel is coated with a coated reactant that has binding affinity for a substrate. Labeled substrate is prepared and is added to the coated reaction vessel along with unlabeled substrate. Depending upon the specific reactant and substrate chosen, the labeled and unlabeled substrate may compete with one another for binding of the coated reactant or the

coated reactant and the unlabeled substrate may compete with one another for binding to the labeled substrate. In either example, the amount of labeled substrate in the soluble portion of the reaction mix is directly proportional to the amount of unlabeled substrate.

In an illustrative example, a competitive assay using E. coli K88 fimbriae receptor is described. Specifically, fluo-fimbriae is prepared by mixing 5 mg NHS-fluorescein in 100 ul DMSO with 10 mg fimbriae in 1 ml PBS (pH 7.2) followed by incubation at room temperature for 3 hr. The receptor is then dissolved in PBS (pH 7.2) to a concentration of 5 ug/ml and 100 ul/well is added to each well of the reaction vessel. The reaction vessel is incubated at 37^0 C for 3 hr, and is then rinsed with PBST, thereby forming a coated reaction vessel. A series of concentrations of the receptor in a buffer (50 ul/well) are added to the wells of the coated reaction vessel. A fixed amount of the fluo-fimbriae (50 ul/well) is added to each well containing the receptor and the control and incubated at 37^0 C for 1 hr. Competitive binding reactions between the immobilized receptor and the free receptor (competitor) to the fluo-fimbriae take place during incubation. That is, the fluo-fimbriae either binds to the immobilized receptor and is removed from the soluble portion or binds to the free receptor and remains in the soluble portion of the reaction mixture. The assay is stopped by removing the soluble portion of the reaction mixture and transferring same to the counting vessel. The fluorescent intensity of the fluo-fimbriae transferred into the wells of the counting vessel is determined using a fluorometer and is directly proportional to the amount of the receptor (competitor).

In another illustrative example, a series of concentration of the

fimbriae or E. coli cell in a buffer (50 ul/well) are added to the wells of a coated reaction vessel prepared as described above. A fixed amount of the fluo-fimbriae (50 ul/well) is added to each well containing the fimbriae or E. coli and control wells and incubated at 37^0 C for 1 hr. Competitive binding reactions between the fimbriae or E. coli (competitor) and the fluo-fimbriae to the immobilized receptor will occur, as described above. The assay is terminated by removing the soluble portion of the reaction mixture and transferring same to the counting vessel. The fluorescent intensity of the fluo-fimbriae transferred into the wells of the counting vessel is determined using a fluorometer and is directly proportional to the amount of the fimbriae or E. coli cells (competitor).

EXAMPLE VIII – COMPETITIVE BINDING ASSAY IN THE PRESENCE OF INHIBITOR

In these examples, the method is carried out as described above except that an inhibitor of binding is included. Thus, the amount of labeled substrate transferred to the counting vessel is directly proportional to the concentration of the inhibitor.

In an illustrative example, the coated reaction vessel is prepared as described in Example VII. A series of concentration of the inhibitor in a buffer (50 ul/well) are added to the wells of the coated reaction vessel. A fixed amount of the fluo-fimbriae (50 ul/well) is added to each well containing the inhibitor and the controls and incubated at 37^0 C for 1 hr. The reactions between the inhibitor and the immobilized receptor for binding to fluo-fimbriae or between the inhibitor and the fluo-fimbriae for binding to the immobilized receptor will occur. The fluorescent

intensity of the fluo-fimbriae transferred into the wells of the counting vessel is determined using a fluorometer and is directly proportional to the amount of the inhibitor (competitor).

While the preferred embodiments of the invention have been described above, it will be recognized and understood that various modifications may be made therein, and the appended claims are intended to cover all such modifications which may fall within the spirit and scope of the invention.

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CLAIMS

1. A method for measuring the activity or concentration of a bioactive molecule comprising:

coating a reaction vessel with a reactant, said reactant being capable of interacting with a bioactive molecule having a biological activity;

adding a sample to the reaction vessel, said sample comprising a known quantity of a detectable label and the bioactive molecule having the biological activity;

incubating the reaction vessel under conditions wherein the reactant and the sample interact;

removing a quantity of detectable label from the sample by binding detectable label to the reactant coated on the reaction vessel;

transferring a soluble portion of the sample from the reaction vessel to a counting vessel ; and

measuring the quantity of detectable label in the counting vessel.

2. The method according to claim 1 wherein the detectable label is selected from the group consisting of: enzyme label; colorimetric label; radioactive label; luminescent label and fluorescent label.

3. The method according to claim 1 wherein the sample is a biological sample.

4. The method according to claim 1 wherein the biological activity is an enzymatic activity.

5. The method according to claim 1 wherein the biological activity is a binding affinity.

6. The method according to claim 1 wherein the sample includes an inhibitor of the biological activity of the bioactive molecule.

7. The method according to claim 1 wherein the sample includes a competitor of the biological activity of the bioactive molecule.

8. The method according to claim 1 wherein the bioactive molecule is selected from the group consisting of: an enzymatic product; an enzyme; a substrate; a lectin; a lectin-binding ligand; a receptor; an inhibitor; a receptor binding ligand; an antigen; and an antibody.

9. The method according to claim 1 wherein the compound is selected from the group consisting of: an enzymatic product; an enzyme; a lectin; a lectin-binding ligand; a substrate; a receptor; an inhibitor; a receptor binding ligand; an antigen; and an antibody.

10. A method for measuring the activity or concentration of a bioactive molecule comprising:

coating a reaction vessel with a reactant, said reactant being capable of interacting with a bioactive molecule, said reactant including a detectable label;

adding to the reaction vessel a sample, said sample including the bioactive molecule having a biological activity;

releasing a quantity of detectable label from the reactant by incubating the reaction vessel under conditions such that the reactant and the detectable label contact the bioactive molecule and interact with the bioactive molecule;

transferring a soluble portion of the sample containing released label from the reaction vessel to a counting vessel; and

measuring the quantity of detectable label in the counting vessel.

11. The method according to claim 10 wherein the detectable label is selected from the group consisting of: enzyme label; colorimetric label; radioactive label; luminescent label; and fluorescent label.

12. The method according to claim 10 wherein the sample is a biological sample.

13. The method according to claim 10 wherein the biological activity is an enzymatic activity.

14. The method according to claim 10 wherein the biological activity is a binding affinity.

15. The method according to claim 10 wherein the sample includes an inhibitor of the biological activity of the bioactive molecule.

16. The method according to claim 10 wherein the sample includes a competitor of the biological activity of the bioactive molecule.

17. The method according to claim 10 wherein the bioactive molecule is selected from the group consisting of: an enzymatic product; an enzyme; a substrate; a receptor; a receptor ligand; an antigen; a lectin; a lectin-binding ligand; a ligand; and an antibody.

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United States Patent [19]

Mallia et al.

[11] Patent Number: 5,538,858

[45] Date of Patent: * Jul. 23, 1996

[54] **RAPID ASSAY FOR RADIOACTIVE DETERMINATION OF PROTEIN KINASE ACTIVITY**

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[73] Assignee: **Pierce Chemical Company**, Rockford, Ill.

[*] Notice: The portion of the term of this patent subsequent to Apr. 8, 2014, has been disclaimed.

[21] Appl. No.: **225,469**

[22] Filed: **Apr. 8, 1994**

[51] **Int. Cl.**[6] **C12Q 1/48**, A61K 51/00

[52] **U.S. Cl.** **435/15**; 435/4, 435/21; 435/194; 436/504; 436/804

[58] **Field of Search** 435/15, 4, 21, 435/194; 436/504, 804; 424/1.1

[56] **References Cited**

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[57] **ABSTRACT**

A rapid radioactive method of measuring enzymatic activity of a protein kinase is disclosed. The method is an improvement to existing methodology which involves phosphorylating a peptide substrate using ^{32}P-ATP, adsorbing the phosphorylated peptide to a solid phase, washing the phase to remove non-adsorbed ^{32}P-ATP, and measuring the radioactivity of the phosphorylated peptide adsorbed to the phase. The disclosed improvement uses a membrane as the solid phase and positions the membrane within a chamber to separate the chamber into a first and second region. Washing is accomplished with centrifugal force; the washed solution being forced through the membrane from the first region into the second region.

12 Claims, 1 Drawing Sheet

FIG. 1

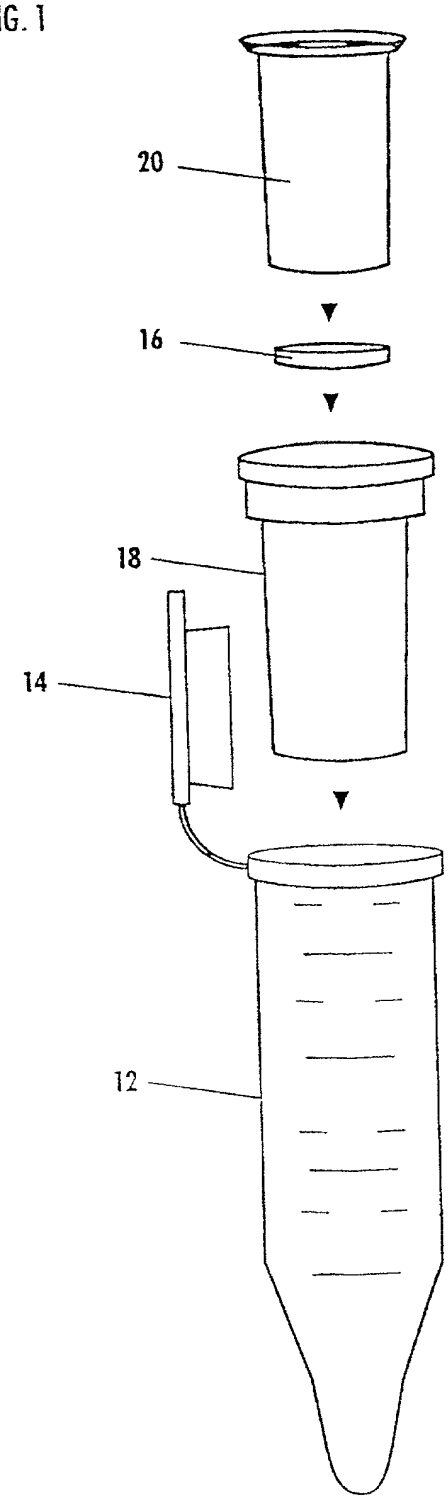
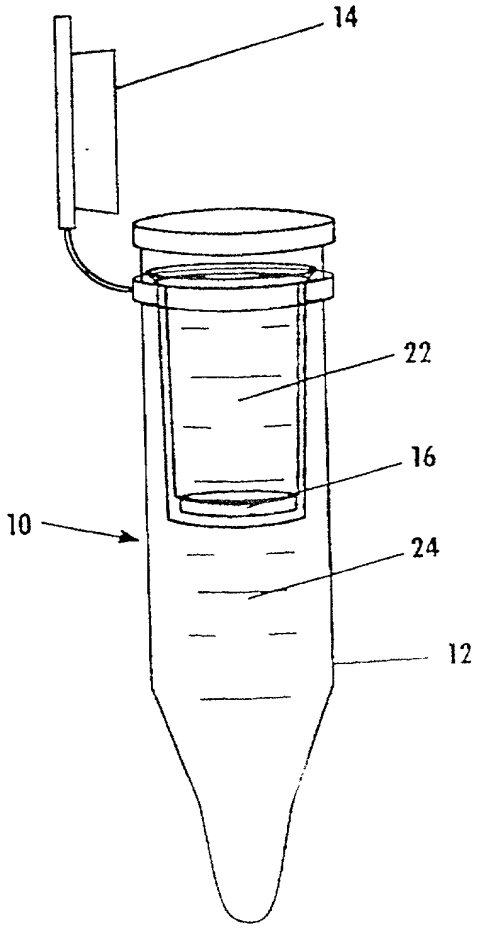


FIG. 2



Docket No.
3027.00020

Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

METHOD FOR MEASURING BIOMOLECULES

the specification of which
(check one)

☒ is attached hereto.

☐ was filed on _____ as United States Application No. or PCT International Application Number _____

and was amended on _____
(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Not Claimed
PCT/CA00/00540	Canada	10/05/00	☐
(Number)	(Country)	(Day/Month/Year Filed)	
			☐
(Number)	(Country)	(Day/Month/Year Filed)	
			☐
(Number)	(Country)	(Day/Month/Year Filed)	

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

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(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
CA2,270,639	May 10, 1999	pending
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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